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Regulation of cell migration by amphoterin XP-001070170

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SUMMARY

Amphoterin, a major form of HMG (high mobility group) 1 proteins, is highly expressed in immature and malignant cells. A role in cell motility is suggested by the ability of amphoterin to promote neurite extension through RAGE (receptor of advanced glycation end products), an immunoglobulin superfamily member that communicates with the GTPases Cdc42 and Rac. We show here that cell contact with the laminin matrix induces accumulation of both amphoterin mRNA and protein close to the plasma membrane, which is accompanied by extracellular export of amphoterin. A role for amphoterin in extracellular matrix-dependent cell regulation is further suggested by the finding that specific decrease of amphoterin mRNA and protein, using antisense oligonucleotides transfected into cells, inhibits cell migration to laminin in a transfilter assay whereas the oligonucleotides in the culture medium have no effect. Moreover, affinity-purified anti-amphoterin antibodies inhibit cell migration to laminin, supporting an extracellular role for the endogenous amphoterin in cell motility. The finding that amphoterin expression is more pronounced in cells with a motile phenotype as compared to cells of dense cultures, is consistent with the results of the cell migration assays. Our results strongly suggest that amphoterin is a key player in the migration of immature and transformed cells.

Key words: Amphoterin, HMG-1, mRNA localization, RAGE, Cell migration

INTRODUCTION

Amphoterin (p30) is a developmentally regulated heparin-binding protein that is abundantly expressed in embryonic brain and in transformed cell lines (Rauvala and Pihlaskari, 1987; Rauvala et al., 1988; Parkkinen et al., 1993). Amphoterin localizes in a diffuse manner in stationary cells but becomes clearly localized to growth cones in neuronal cells and correspondingly to leading edges in non-neuronal cells when extension of cytoplasmic processes is stimulated by matrix proteins like laminin (Rauvala et al., 1988; Merenmies et al., 1991; Parkkinen et al., 1993). Exogenously added matrix-bound amphoterin is highly active in enhancing neurite outgrowth in brain neurons (Rauvala and Pihlaskari, 1987) and various cells are also able to deposit amphoterin to the cell matrix (Merenmies et al., 1991), where its amount is regulated by plasmin activity of the culture medium (Parkkinen et al., 1993). Extracellular roles in neurite outgrowth, in neuron-glia interactions and in early phases of cell differentiation have been suggested for amphoterin (Rauvala and Pihlaskari, 1987; Rauvala et al., 1988; Daston and Ratner, 1991, 1994; Hori et al., 1995; Melloni et al., 1995). Very recently, amphoterin (or HMG-1; Bianchi et al., 1989) has been identified as a secreted late mediator of endotoxin shock in mice, supporting the extracellular function (Wang et al., 1999). Amphoterin was also found to be the endogenously occurring ligand that binds to the extracellular moiety of RAGE, the receptor for advanced glycation end products (Hori et al., 1995). The neurite outgrowth induced by RAGE-amphoterin interaction involves Rac and Cdc42 (Huttunen et al., 1999). These results suggest that amphoterin possesses an extracellular function though it lacks a secretion signal (Merenmies et al., 1991).

Cell migration plays an important role in both normal physiology and disease. The process of cell migration involves a dynamic interaction between the cell and the extracellular environment. For example, laminin through its binding to integrin on the cell surface has been demonstrated to be a permissive substrate for glioma migration and invasion (Uhm et al., 1999). In our early studies, amphoterin has been shown to localize at the leading edges of transformed cells grown on laminin, and anti-amphoterin antibodies in the medium have been able to inhibit neurite outgrowth on laminin (Merenmies et al., 1991).

In order to further understand the sorting mechanism and the function of amphoterin in motile cells, we have in this study followed the distribution of amphoterin mRNA and protein in C6 glioma cells. We show that an essential step in the sorting of amphoterin to the cell processes already occurs at the mRNA level and that the protein remains close to the mRNA in cells. In addition, laminin when applied locally via microbeads, is able to regulate the distribution of amphoterin mRNA and protein. Further, inhibition of amphoterin mRNA by specific anti-sense oligonucleotides decreases significantly haptotactic migration of several transformed cell lines to laminin, in agreement with the

localization that suggests a role in cell motility. Inhibition of cell migration by anti-amphoterin antibodies suggests an extracellular role in the process of cell migration. In conclusion, we propose that cell-matrix contact induces transport of amphoterin mRNA to the cell processes, where it is translated. The protein is then exported to the extracellular space, where it enhances cell migration by binding to the cell surface receptor.

MATERIALS AND METHODS

Cell culture

N18 mouse neuroblastoma cells, C2C12 mouse myoblast cells, C6 rat glioma cells, B16 mouse melanoma cells and HT1080 human fibrosarcoma cells were maintained under standard culture conditions. After desired culture periods on laminin or plastic dishes, the cells were fixed with 4% paraformaldehyde/0.05% glutaraldehyde in PBS and stored at 4°C. Puromycin (200 μg/ml) and cycloheximide (5 μg/ml) were used to inhibit protein synthesis.

Probes

For in situ hybridization, linearized Bluescript KS plasmid containing the full-length coding region (650 bp) of rat amphoterin (Merenmies et al., 1991) was transcribed in vitro using DIG RNA Labeling kit (Boehringer Mannheim, Mannheim, Germany) as previously described (Fages et al., 1998). For northern blotting, the full-length coding sequence of amphoterin and the *Hinc*II fragment (850 bp) of rat β-actin (Fages et al., 1998) were labelled with [α-32P]dCTP with Ready To Go Kit (Amersham Pharmacia, Uppsala, Sweden) according the manufacturer's instructions.

Antibodies

The anti-amphoterin antibodies used in the present study were produced against the synthetic peptide KPDAAKKGVV (residues 166-175 of the amphoterin sequence, antipeptide II) and affinity-purified using the same peptide (Merenmies et al., 1991). These antibodies were previously shown to bind to both purified native and denatured amphoterin, and they detected amphoterin specifically in western blotting of SDS-solubilized N18 cells and rat brain (Merenmies et al., 1991). The antibodies were also shown to discern amphoterin from other HMG1-type proteins (Parkkinen et al., 1993). Antibodies against the whole recombinant amphoterin were also used in western blotting and immunocytochemistry (Parkkinen et al., 1993). Rabbit IgG (10-25 μg/ml) used as a control was purified from nonimmune rabbit sera with protein A-Sepharose (Pharmacia, Uppsala, Sweden) as recommended by the manufacturer. The anti-β-actin antibody was purchased from Sigma.

In situ hybridization

In situ hybridization was performed as previously described (Fages et al., 1998). In addition, a protocol using digoxigenin-labelled amphoterin RNA probe and tyramide signal amplification was used to amplify the sensitivity of the detection as previously described (Punnonen et al., 1999). The mRNA signals were analyzed by three-dimensional imaging microscopy (BioRad).

Immunocytochemistry and simultaneous detection of mRNA and protein

For confocal and standard immunofluorescence microscopy the fixed cells, permeabilized with methanol for 5 minutes, were blocked with 10% skimmed milk powder in PBS for 1 hour, and immunostained with the anti-amphoterin antibodies (5 μg/ml) or non-immune IgG (10 μg/ml) for 1 hour. Bound antibodies were detected with affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.), diluted 1:100. Cells were mounted with 50% glycerol in veronal buffer, pH 8.6, containing 2.5% Dabco (Sigma). For double immunostaining with anti-β-actin (Sigma) and anti-amphoterin antibodies, the fixed cells were blocked first in 50 mM NH_4Cl in PBS for 10 minutes, then with 3% BSA for 15 minutes. The two proteins were detected sequentially. First, amphoterin was detected as described above. Then, β-actin was revealed using anti-β-actin followed by affinity-purified tetramethyl rhodamine (TRITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). All dilutions were prepared in 3% BSA/PBS. Cells were mounted in 30% glycerol in Moviol, pH 7, containing 50 mg/ml Dabco.

For surface staining of living cells, N18 cells were washed with PBS and incubated in 3% BSA/PBS on ice for 10 minutes. Then primary antibodies were added to the cells for 60 minutes on ice. After washing, the cells were fixed in 4% paraformaldehyde/PBS for 20 minutes and 50 mM NH_4Cl was used to quench the free aldehyde groups. The cells were then double-immunostained to detect amphoterin and β-actin (see above).

To detect amphoterin mRNA and protein simultaneously, in situ hybridization using tyramide amplification was performed first, followed by the immunocytochemistry as above. Amphoterin antibodies were then detected with affinity-purified TRITC-conjugated goat anti-mouse IgG. Confocal laser fluorescence (BioRad MRC1024 confocal system) microscope was used to analyze the results.

Ribosomes were labeled with a rabbit antibody against the 60S ribosomal subunit (a kind gift of Dr John Hesketh, Rowett Research Institute, Bucksburn, Aberdeen, UK), and protein disulfide isomerase was labeled with a mouse monoclonal antibody 1D3 (a kind gift of Dr Stephen Fuller, European Molecular Biology Laboratory, Heidelberg, Germany). The primary antibodies were detected with goat anti-rabbit-FITC or goat anti-mouse-LRSC (Jackson ImmunoResearch Laboratories). The cells were embedded in Mowiol containing Dabco as an antifading agent, and examined with the confocal microscope.

Scanning electron microscopy

Living cells grown on glass coverslips were immunolabeled with anti-amphoterin on ice as explained above, and after fixation in 4% paraformaldehyde, the primary antibody was detected using swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) and protein A coupled to 10 nm gold (University of Utrech, the Netherlands). The cells were then post-fixed in 2.5% glutaraldehyde in PBS. The signal was amplified using Intense M silver enhancement kit (Amersham Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions. After dehydration with ethanol, the cells were treated with hexamethyldisilazane (Fluka, Buchs, Switzerland) for 5 minutes and then air-dried. Finally, the cells were coated with a thin layer of platinum and examined by scanning microscope (Zeiss DSM 962, Germany) using the back scatter electron detector to visualize gold and silver.

Bead assays

Polystyrene beads (diameter 4.5 μm or 10 μm, Polysciences, Inc., Warrington, PA, USA) were washed with 95% ethanol and incubated with laminin or lentil lectin at a concentration of 100 μg/ml, expected essentially to saturate the bead surface, in PBS for 1 hour at room temperature as previously described (Fages et al., 1998).

Electrophoretic methods

Total RNA was isolated from the dishes as above. RNeasy Total RNA kit (Qiagen, Hilden, Germany) was used to isolate RNA from confluent cells (2 days in 10% horse serum in DME) and from cells growing under standard culture conditions. Northern analyses were performed by standard procedures (Sambrook et al., 1989). The nylon filters were probed overnight (50% formamide, 60°C) and washed twice with 2× SSC/0.1% SDS for 10 minutes, and then twice with 0.1× SSC/0.1% SDS for 15 minutes at 60°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out in gradient gels and the proteins were transferred to nitrocellulose (Towbin et al., 1979). The membrane was then incubated overnight with 1% BSA in 20 mM Tris-HCl, pH 7.8, 500 mM NaCl. Immunostaining was carried out with affinity-purified peptide antibodies or with anti-rec-Atn at 0.4 μg/ml for 1 hours. The

bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reaction (Amershan Corp.)

Antisense oligodeoxynucleotides

C-5 propynylpyrimidine-2′-deoxyphosphorothioates-modified ODNs (Wagner et al., 1993) were synthesized (Perkin Elmer, Applied biosystems DNA synthesizer 394, Foster City, CA, USA) and purified by reversed-phase HPLC (Bengström and Paulin, 1991). The C-5 propynylpyrimidine modification of the ODNs has been shown to greatly enhance affinity for RNA, imparted by the C-5 propynyl moiety (Wagner et al., 1993). Each ODN (0.5 μM) was premixed with Tfx™ reagent (Promega, Madison, WI, USA) and added to C6, N18 or HT1080 cells in the DMEM medium. After 2 hours, the cells were washed and incubated for an additional 12 hours in DMEM supplemented with 10% FBS. The transfection efficiency was measured using a 5′-fluoresceinated ODN. Total RNA was isolated as described above. The RNA was quantified spectrophotometrically, and 10 μg of total RNA was analyzed on each lane. Northern analysis was performed using the full-length coding sequence of the amphoterin cDNA. The 18-nt antisense ODN, corresponding to the 3′ coding region, was 5′-UUCAUCUUCGUCUUCCUC-3′ (positions 730-748) and the nonsense ODN, having the same nucleotide composition, was 5′-CCUACUCUUUCU-GUCUCU-3′.

Neurite outgrowth and transfilter migration assays

After transfection, the cells were grown on laminin (5 μg/ml) for 6 hours or overnight at 37°C in order to induce neurite outgrowth. The cells were then fixed with cold methanol and stained with 0.05% Toluidine Blue. After the staining, the cells were examined under a light microscope using 20× objective and four fields were counted.

The migration assay was carried out in Transwell® chambers (pore size 12 μm; Corning Costar) essentially as described previously (Imai et al., 1998). Under the conditions used, 80-90% of the added cells migrated to the lower surface of the filter (Imai et al., 1998). Laminin was coated to the lower surface of the filter at 5 μg/ml for 1 hour at room temperature. Cells were placed in the upper chamber at 20×10^4 cells/ml, and cultured for 6 hours or overnight. After fixing with cold methanol, the cells were stained with 0.05% Toluidine Blue and removed from the upper surface of the filter. The cells that had migrated to the lower surface were then examined under a light microscope. Four independent fields per filter were counted for the number of migrated cells.

Secretion assay

N18 cells were grown on laminin-, HB-GAM- or BSA-coated plates for 3 hours, 6 hours or overnight in the presence of aprotinin (10 μg/ml; Sigma). The cells were then washed briefly and incubated for 1 minute with 10 μg/ml of low molecular mass (LMW) heparin (Sigma) in the culture medium to detach amphoterin from the cell surfaces. Secreted amphoterin was then detected by western blotting (see above).

RESULTS

Amphoterin mRNA and protein localize peripherally in cells grown on laminin

Previous studies have shown that transformed cells express abundantly amphoterin mRNA and protein in comparison to their normal counterparts and show a peripheral localization for amphoterin protein when they start to extend processes (Merenmies et al., 1991; Parkkinen et al., 1993). In order to compare the protein and the mRNA distribution, we performed an in situ hybridization and immunocytochemistry using C6 cells grown for 3 hours on laminin. The localization of the mRNA and

Fig. 1. Amphoterin mRNA (A, green) and protein (A, red) localize at the leading edge in C6 cells (A, arrowhead). C6 cells were grown on laminin (10 μg/ml) for 3 hours in a serum-free medium before fixation. In situ hybridization was then performed with a digoxigenin-labeled amphoterin anti-sense probe followed by immunostaining with anti-amphoterin antibodies. Immunofluoresescence of ribosomal 60S subunit shows a strong staining for ribosomes in cell processes (B, arrowheads), suggesting a local mode of translation in C6 cells. Laminin induces the localization of amphoterin protein (C) and mRNA (D). C6 cells were incubated with the laminin-coated beads (b) for 15 minutes. Almost 40% of the laminin-coated beads are able to localize amphoterin protein as well as the mRNA (E), whereas lentil lectin-coated beads have almost no effect (10.5%; F). In E and F, each value represents the mean ± s.d. calculated from 12 randomly selected microscopy fields in 3 independent experiments. Bars, 50 μm (A-D).

protein resemble each other (Fig. 1A). Amphoterin mRNA is detected in the membrane extensions that are stained for the protein (Fig. 1A). Nonimmune IgG used as a control for anti-amphoterin antibodies gave only diffuse background staining in all staining procedures used (not shown). Numerous ribosomes were detected both in the varicosities and the leading edge of C6 cells (Fig. 1B, arrowheads). Moreover, puromycin or cycloheximide, inhibitors of translation, did not have any effect on the localization of the mRNA (not shown). This suggests that translation and localization of amphoterin mRNA occur independently.

Localization of amphoterin mRNA and protein is regulated by laminin

Recent studies have shown that mRNA localization is regulated through transmembrane signalling upon cell-matrix interaction (Fages et al., 1998; Chicurel et al., 1998). Localization of amphoterin mRNA in response to laminin (Fig. 1A) could be due to an indirect consequence of process outgrowth. Therefore, laminin-coated beads were applied to C6 cells for 15 minutes. As shown in Fig. 1D, local presentation of laminin was very efficient in localizing amphoterin as the majority of the mRNA signal was concentrated around the beads, irrespectively of the position of the beads on the surface of the cell soma or of the growing processes. Moreover, the same proportion of beads induced the localization of mRNA and protein, suggesting a similar regulatory mechanism (about 40%; Fig. 1C,E). However, lentil lectin-coated beads had little effect in localizing the mRNA, indicating that not all beads binding to the cell surface possess mRNA-localizing activity (Fig. 1F).

Amphoterin is exported to the extracellular space from cells extending processes on laminin

Laminin-coated beads are rather rapidly (within 20-30 minutes) endocytosed by the cells, excluding this method for studying whether cell-matrix contact might promote extracellular export of amphoterin. We therefore investigated the distribution of amphoterin in the medium and in cells using N18 cells that display a motile phenotype on laminin-coated substrate but do not extend processes on some other matrix proteins, like HB-GAM (for a review, see Rauvala and Pe... 1997), despite their strong adhesion. Interestingly, western blotting showed a consistent increase in the amount of amphoterin in the extracellular medium when cells were plated on laminin as compared to HB-GAM (Fig. 2A) or BSA (not

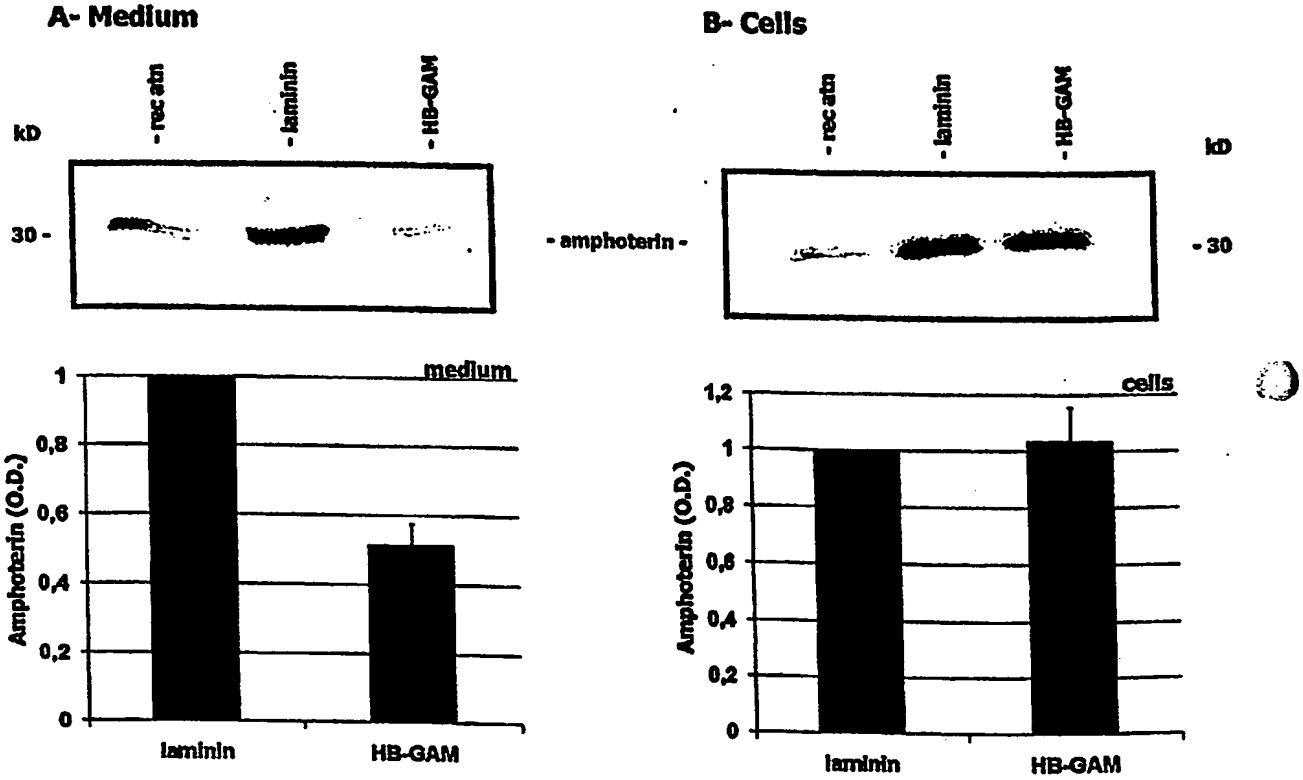

Fig. 2. Secretion of amphoterin accompanies process extension. N18 cells were grown overnight on laminin- (10 μg/ml) or HB-GAM- (10 μg/ml) coated wells in the presence of aprotinin (10 μg/ml). The cells adhere and extend processes on laminin. Despite of their strong adhesion, N18 cells do not grow processes on HB-GAM. The medium was then removed and the cells were incubated for 1 minute in the culture medium with heparin (10 μg/ml) to detach amphoterin from the culture. Without the heparin treatment, no or only very low amounts of amphoterin were detected in the extracellular space by western blotting. The proportion of the medium loaded per lane corresponds to 10 μg of total cellular protein (the same proportion of the culture as was loaded from the cell lysate). The cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl,1% NP-40,1 mM PMSF and 2 μg/ml aprotinin, and western blotting was performed using 10 μg of total cellular protein per lane. Recombinant amphoterin (50 ng) was used as a control. Relative intensity of the amphoterin band (amphoterin OD) from cultures on laminin or HB-GAM represents the mean ± s.d. from four independent experiments.

shown). The 30 kDa band found in the extracellular space was detected by antibodies against recombinant amphoterin (Fig. 2A). It was also detected by the five different affinity-purified anti-peptide antibodies (not shown), that cover the amphoterin sequence from the N-terminal region close to the C-terminal region (Parkkinen et al., 1993). These results discount the possibility that the 30 kDa band detected in the extracellular space could be due to a protein that only crossreacts with the antibodies. No changes in the cellular level of amphoterin were observed in the experiments (Fig. 2B). Amphoterin is thus exported to the extracellular space from process-extending cells, whereas only cell binding to the matrix does not significantly enhance extracellular expression.

As expected from the localization of amphoterin studied previously (Daston and Ratner, 1991; Merenmies et al., 1991; Parkkinen et al., 1993) and in the present study, double-immunostaining of amphoterin and β-actin revealed a similar staining at the cell periphery when studied at light microscopy level. However, a clear difference was found in the localization at the plasma membrane; both in living (Fig. 3A) and in permeabilized cells (Fig. 3B), amphoterin was stained in most cells as patches at the plasma membrane. Double-immunostaining showed that these patches do not contain β-actin (Fig. 3A, B), suggesting that the release of amphoterin is not due to nonspecific cell damage. The extracellular patchy staining was detected both by anti-recombinant amphoterin antibodies (not shown) and by the anti-peptide antibodies (Fig. 3) that bind specifically to amphoterin in SDS-solubilized N18 cells (Merenmies et al., 1991) and distinguish it from closely related variants of the protein (anti-peptide II, Parkkinen et al., 1993). Binding of these antibodies to the cell surface was abolished by the corresponding synthetic peptide in solution (Fig. 3C). Occurrence of amphoterin at the cell surface was also clearly visualized by immunoscanning electron microscopy (Fig. 3D). Double immunostaining of amphoterin and PDI (protein disulfide isomerase), which is a soluble ER-lumen enzyme, showed no colocalization, suggesting that amphoterin does not enter the classical secretory pathway (not shown).

Inhibition of amphoterin synthesis by antisense ODN decreases haptotactic cell migration to laminin

Localization of amphoterin to the processes of migrating cells suggests a role in cell motility. We therefore studied whether a specific decrease of amphoterin mRNA in cells would influence cell motility. Decrease of amphoterin mRNA, but not β-actin mRNA, was achieved using C-5 propynylpyrimidine-2-deoxyphosphorothioate-modified amphoterin antisense ODNs transfected into cells (Fig. 4A). In contrast, no effect was observed with the same oligonucleotides in the culture medium, or when sense or nonsense ODNs were used (Fig. 4A). In agreement with the reported high mRNA affinity of the type of ODNs used (Wagner et al., 1993), rather low concentrations (400-500 nM) of the transfected ODNs strongly reduced amphoterin mRNA (to 15-20% of the control level; see Fig. 4A). Western blotting showed that the antisense effect also decreased the content of amphoterin protein (Fig. 4B).

A morphological effect was observed in C6 glioma cells, into which the inhibitory oligonucleotides (see above) had been transfected. Thus, nearly all (90-100%) cells extended long cytoplasmic processes when grown for 6 hours on laminin matrix, whereas most ODN-transfected cells displayed a round morphology (not shown). A decrease to 40% of the controls (non-transfected cells, or cells transfected with sense or nonsense ODNs) was observed in the proportion of process-extending cells, when 500 nM amphoterin anti-sense ODNs were transfected into C6 cells.

Since these results suggested that a decrease of amphoterin mRNA affects the migratory response of cells, we studied the effect of the different ODNs further using a haptotactic transfilter assay. As shown in Fig. 4C-D, transfection of the amphoterin antisense ODNs into C6 cells inhibited cell migration to laminin to about 40% of control values. The inhibitory effect on cell migration correlates to the reduction of amphoterin mRNA; for example, no effect on cell migration was observed for the ODNs used in the culture medium (Fig. 4D). A similar migration inhibition was observed in other cell types tested (N18 and HT1080; data not shown).

Anti-amphoterin antibodies inhibit cell migration

Previous studies have shown that anti-amphoterin antibodies inhibit, in a reversible manner, neurite outgrowth on laminin (Merenmies et al., 1991). To investigate the mechanism by which amphoterin regulates cell migration, anti-amphoterin antibodies were added in the medium during the haptotactic transfilter assay using N18 (Fig. 5) or C6 cells (not shown). Cell migration to laminin was inhibited in a dose-dependent manner, whereas nonimmune IgG did not display a significant effect (Fig. 5). In C6 cells, a similar situation was observed; almost 80% of the cell migration was inhibited (19±10.2% migrated cells as compared to the control without added antibodies). These results strongly suggest that amphoterin acts extracellularly during cell migration.

Amphoterin mRNA is strongly expressed in migrating cells but is downregulated during cell-to-cell contact

When cells become confluent the amount of amphoterin mRNA is reduced compared to cells that display a motile phenotype. Both in situ (not shown) and northern hybridizations (Fig. 6) performed with C6 cells demonstrate this. When confluency is reached, the amphoterin mRNA diminishes to 40% of the control level found in migrating C6 cells (Fig. 6).

DISCUSSION

Amphoterin was shown to be highly enriched in the processes of immature and malignant cells (Parkkinen et al., 1993). However, the amphoterin sequence lacks a secretion signal and the same sequence was cloned for HMG-1 (Bianchi et al., 1989; Merenmies et al, 1991). The sequence consists of a strong polycationic N-terminal part followed by a polyanionic tail (hence the designation amphoterin) (Merenmies et al., 1991). These features apparently explain, at least in part, the highly adhesive nature of amphoterin (Rauvala and Pihlaskari, 1987).

The structural characteristics and the peripheral localization of amphoterin are however controversial. Previous studies on the distribution of the endogenous protein in vitro (Rauvala et al., 1988; Merenmies et al., 1991) and in vivo (Daston and Ratner, 1991; Hori et al., 1995; Milev et al., 1998; Nair et al.,

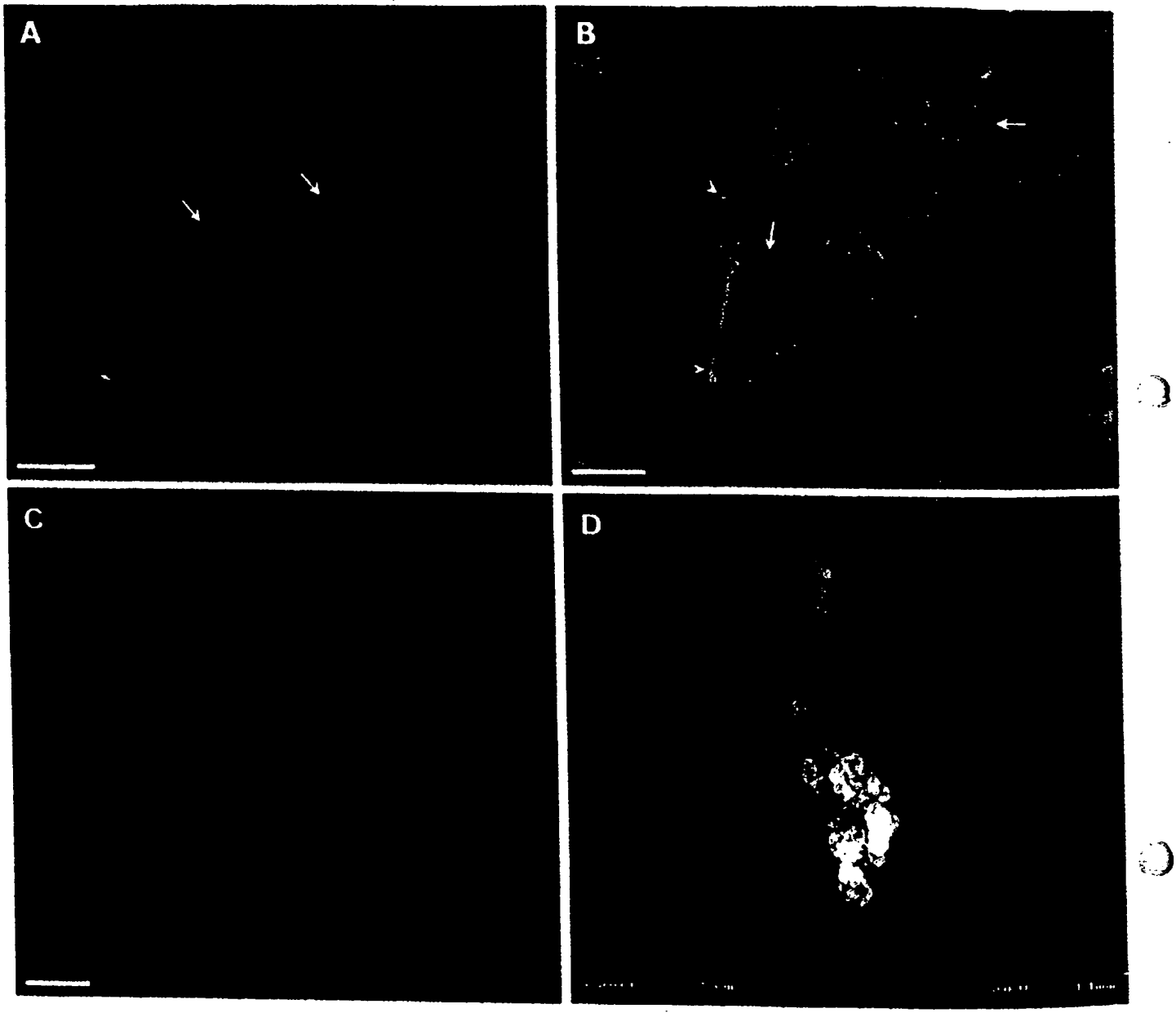

Fig. 3. Localization of amphoterin on the surface of N18 cells. The cells were cultured on laminin-coated substrate for 3 hours. Primary antibodies (anti-peptide II) were applied to living cells on ice (A,C,D), or to fixed and methanol-permeabilized cells (B). (A,B) Double immunofluorescence of amphoterin (green) and β-actin (red). Amphoterin is found in extracellular patches (arrows), which show no labeling for β-actin even after permeabilization (B). A shows no actin labeling, which indicates that the cells were intact during the incubation with primary antibodies. The yellow staining shows the colocalization of the proteins after permeabilization (B) in cortical cytoplasm, in filopodia and growth cones (arrowheads). In (C), the anti-amphoterin antibody was incubated with the peptide antigen (190 μM) in the presence of 3% BSA overnight, before it was applied to the cells. Scanning electron microscopy was used to study the appearance of amphoterin-containing extracellular patches (D). The field shows a portion of a neurite with amphoterin-labeled vesicle-like structures. A-C were reconstructed by superimposing 0.5 μm optical sections taken through the depth of the monolayer. Bars, 20 μm (A-C), 5 μm (D).

1998) have indicated a peripheral and extracellular distribution. During the last few years the idea has emerged that mRNA transport to specific locations in the cell may be an important mechanism in molecular sorting (for reviews, see Wilhelm and Vale, 1993; St Johnston, 1995; Steward, 1995). The localization of a mRNA would then allow the protein in question to be synthesized close to the presumable site of function. In the present paper, we show that the localization of the amphoterin protein is tightly connected to the localization of the mRNA, suggesting that the mRNA is locally translated. These results agree with the ultrastructural localization of the amphoterin mRNA (Punnonen et al., 1999). The occurrence of ribosomes in the processes of the cells studied in the present paper is compatible with a local mode of translation.

We show in the present paper that amphoterin is detected as patches at the cell surface, and these do not contain β-actin. Amphoterin has been implicated in several phenomena like neurite outgrowth and neuron-glia interaction, in early phases of cell differentiation and recently in endotoxemia (Rauvala and Pihlaskari, 1987; Rauvala et al., 1988; Daston and Ratner, 1991; Hori et al., 1995; Melloni et al., 1995; Wang et al., 1999). Since amphoterin lacks a classic-type secretion signal, its extracellular expression is not well understood. In the present paper we show that accumulation of amphoterin to the extracellular space accompanies process extension due to laminin, but is much lower in cells that only bind to the cell matrix without extending processes. Interestingly, the extracellular export during process extension correlates with the laminin-induced localization of the mRNA. Unfortunately, we could not observe extracellular amphoterin around the beads due to the bead invagination into the cells after a 30 minute incubation. However, we have shown by western blotting that the level of amphoterin in the cell lysate does not change in cells grown on laminin or HB-GAM; only the extracellular level of the protein is dependent on matrix interactions that enhance process outgrowth. Taken together, we speculate that the laminin-induced localization of the mRNA and the consequent local translation of amphoterin facilitate the extracellular export.

Monoclonal anti-amphoterin antibodies have been used to show an effective transport to the cell surface under conditions that do not involve cell damage and appear to depend on

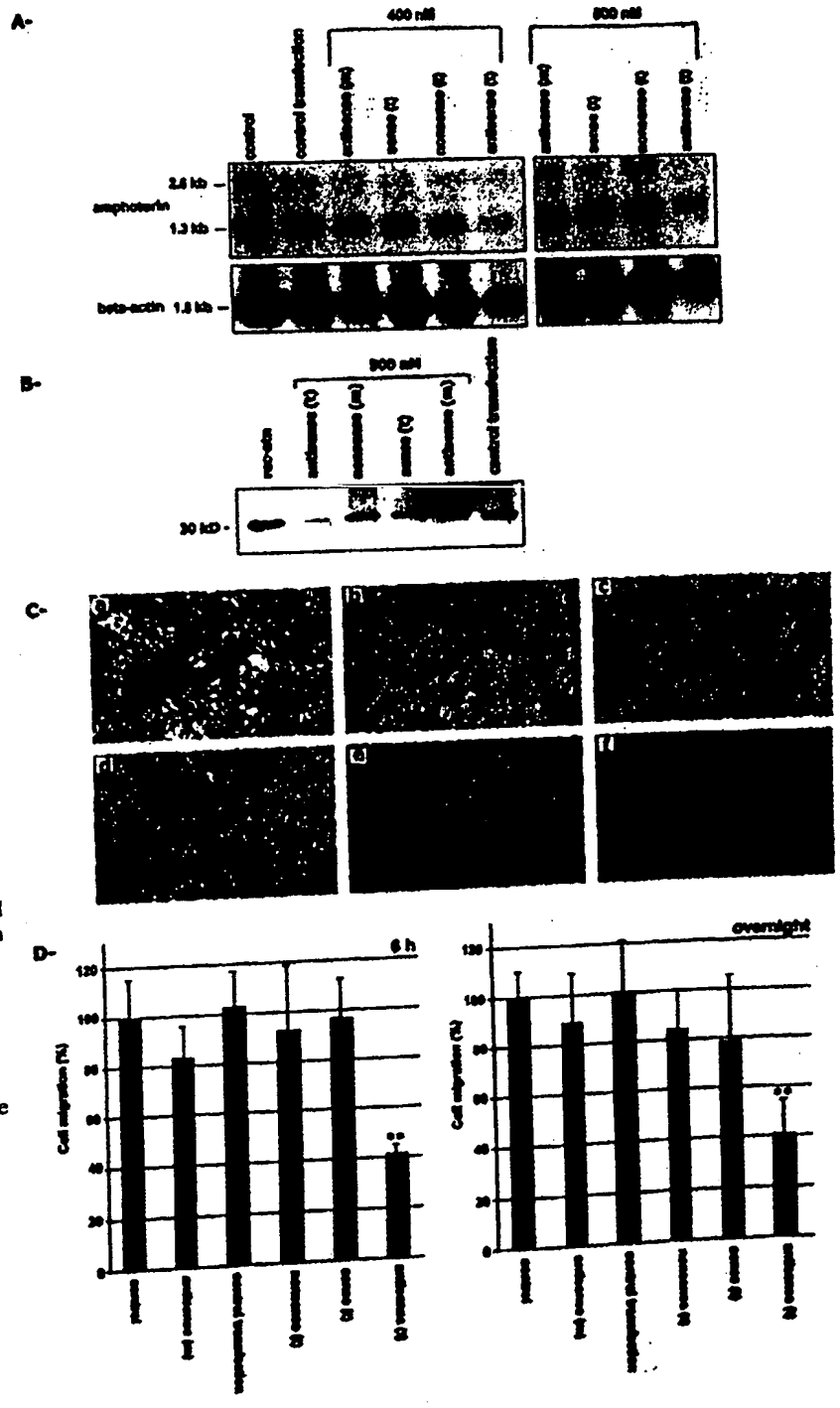

Fig. 4. Downregulation of amphoterin expression inhibits cell migration. For northern analysis (A), C6 cells were transfected overnight with two different concentrations (400 nM and 500 nM) of C-5 propynylpyrimide-2′-deoxyphosphorothioate-modified antisense ODNs (antisense (t)). The controls were: untreated cells (control); control transfection without ODNs (control transfection), antisense added into the medium without transfection (antisense (m)), sense ODN transfected (sense (t)) and nonsense ODNs transfected (nonsense (t)). Comparison of the band intensity (OD) between antisense (t) and antisense (m) shows a specific decrease of the amphoterin mRNA level in the cells transfected with the antisense ODN to 20% and 15% of the control value at 400 nM and 500 nM antisense ODNs, respectively. No change is observed for the β-actin mRNA in the same experiments. For western blotting of protein expression (B) in C6 cells, 500 nM ODNs were used. The level of the amphoterin protein decreases to 21±3% of the control value, calculated from three independent experiments. (C,D) The effect of the antisense transfection on cell migration, using a transfilter assay. The lower surface of the transwell filter was coated with laminin (5 μg/ml), and the C6 cells that migrated from the upper chamber to the lower chamber were studied under the microscope after 6 hours (not shown) or 20 hours (Ca-f). a, the untreated cells; b, the control transfection without ODNs; c, cells incubated with the antisense ODN without transfection reagent; d, cells transfected with nonsense ODN; e, cells transfected with the sense ODN and f, cells transfected with the antisense ODN. (D) Counts of the migrated cells after 6 hours and 20 hours using 500 nM ODN concentration. (D) Each value represents the mean ± s.d. calculated from 24 randomly selected microscopic fields in six independent experiments. $^{**}P<0.01$ (ANOVA test).

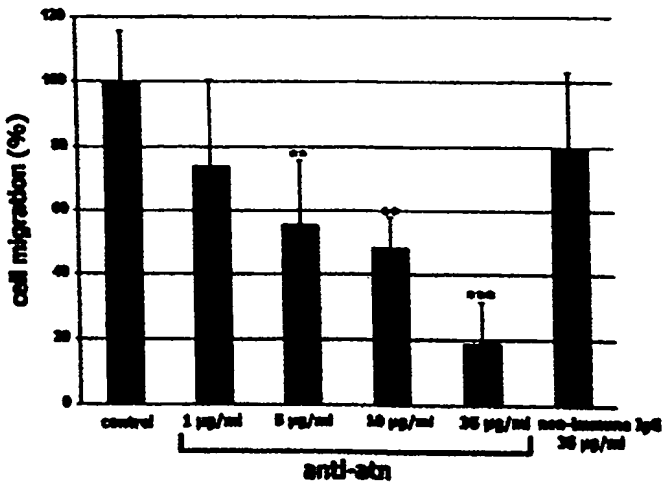

Fig. 5. Anti-amphoterin antibodies inhibit cell migration in a dose-dependent manner. Affinity-purified anti-amphoterin antibodies (anti-peptide II; see Materials and Methods) and non-immune IgG were dialyzed against PBS and then against the assay medium (DME-BSA). N18 cells were incubated for 5 minutes with the antibodies before the migration assay. The lower surface of the transwell filter was coated with laminin (10 μg/ml) for 1 hour and then the cells were applied. After 5 hours, the cells were fixed with cold methanol and stained with Toluidine Blue. The migrated cells were counted under a 20× microscopic objective. Each value represents the mean± s.d. calculated from 12 randomly selected microscopic fields in three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$(ANOVA test).

protein kinase C and influx of calcium ions into the cell (Sparatore et al., 1996; Passalacqua et al., 1997, 1998). Very recently, cytokines such as TNF and IL-1 were shown to induce extracellular export of amphoterin both in vitro and in vivo (Wang et al., 1999). As shown in the present study, cell-matrix contact is also able to enhance extracellular export of amphoterin. It is conceivable that local synthesis of amphoterin close to the plasma membrane would facilitate extracellular export. It would be interesting to learn, whether mRNAs of other proteins lacking a secretion signal but having apparent extracellular roles are also targeted close to the plasma membrane.

Amphoterin is a ubiquitous protein but its expression level is downregulated during cell and tissue differentiation (Rauvala and Pihlaskari, 1987; Parkkinen et al., 1993). In our previous study, we showed that the expression level of amphoterin is very high in transformed cell types as compared to their non-transformed counterparts (Parkkinen et al., 1993). Therefore, the high expression of amphoterin in malignant cells may be an important factor in the regulation of cell motility during tumor invasion. In order to address the question of whether the expression level of amphoterin affects cell motility, we have worked out a method to specifically decrease the level of amphoterin expression in cells. To this end, we have used a recently described modification of oligonucleotides (Wagner et al., 1993) that enhances the binding affinity and therefore makes it possible to use low concentrations of the oligonucleotides. When transfected into several cell types, the antisense oligonucleotides reduce the amount of amphoterin mRNA to a level similar to that

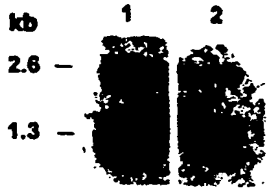

Fig. 6. Expression of amphoterin mRNA is downregulated upon cell-to-cell contact. The gel was loaded with samples (5 μg each) from confluent (lane 1) and exponentially growing (lane 2) C6 cells. When confluency is reached, the amphoterin signal (band intensity of the 1.3 kb amphoterin mRNA) decreases to 40% of that found in spreading cells.

observed during cell density-dependent downregulation of amphoterin expression. The antisense treatment indeed inhibits migration of various cell types, as is demonstrated in a haptotactic transfilter assay. Since oligonucleotides have a propensity to bind to the cell surface, which causes nonspecific effects in cell behavior, we have used oligonucleotides in the culture medium as one way to control the specificity of the effect. From these experiments, we conclude that the effect of the antisense treatment used in this study is specifically due to reduction of the target mRNA. In addition, we have shown that anti-amphoterin antibodies added in the medium significantly reduce cell migration, indicating an extracellular role for amphoterin in cell motility. Taken together, our results strongly suggest a regulatory role of amphoterin in cell migration.

RAGE (receptor of advanced glycation end products), an immunoglobulin superfamily member with closest homology to N-CAM (neural cell adhesion molecule), has been recently shown to bind amphoterin (Hori et al., 1995). RAGE is the receptor for the advanced glycation end products (AGEs) that accumulate in diabetes and during senescence, and it is also suggested to act as a receptor of the β-amyloid (Aβ) peptide in the neurodegenerative mechanism of Alzheimer's disease (Yan et al., 1996) as well as that of EN-RAGE, a member of the S100/calgranulin family, in chronic inflammation (Hofman et al., 1999). Since RAGE is already expressed during embryonic development, physiological RAGE ligands have been sought from tissue extracts, which has resulted in the isolation of amphoterin as a ligand that binds to the ectodomain of RAGE (Hori et al., 1995). Interestingly, amphoterin competes with AGEs, Aβ and EN-RAGE for binding to the RAGE ectodomain (Hofman et al., 1999).

The soluble ectodomain of RAGE has been shown to inhibit neurite outgrowth on amphoterin matrix (Hori et al., 1995). Further, we have very recently shown that transfection of cells with full-length RAGE, but not the cytosolic domain-deleted RAGE, strongly enhances extension of filopodia and neurites in cells grown on matrix coated with recombinant amphoterin (Huttunen et al., 1999). The amphoterin/RAGE-mediated process extension depends on the Rho-family GTPases Cdc42 and Rac, but not on Rho or Ras (Huttunen et al., 1999). The amphoterin/RAGE/GTPase pathway thus provides one mechanistic explanation for the role of amphoterin in cell migration. It is also noteworthy that amphoterin binds to cell surface proteoglycans (Salmivirta et al., 1992; Milev et al.,

1998) and sulfoglycolipids (Nair et al., 1998) that may have a role in amphoterin-induced cell migration.

In conclusion, we suggest a model according to which amphoterin is targeted to the cell processes at the mRNA level, synthesized locally close to the plasma membrane and released to the extracellular space. Amphoterin then binds to the RAGE protein and sulfated glycan epitopes in proteoglycans and glycolipids, which may enhance invasive process extension in metastatic cells. During motile phenomena amphoterin may be released by the migrating cell itself ('autocrine mechanism') or by the neighboring cell ('paracrine mechanism'). It is also noteworthy that amphoterin binds both plasminogen and t-PA (tissue-type plasminogen activator), which results in degradation of amphoterin and strong enhancement of proteolytic activity (Parkkinen and Rauvala, 1991; Parkkinen et al., 1993), which may facilitate cell invasion during metastasis. The RAGE-mediated effect on process extension would thereby be terminated by degradation of amphoterin by plasmin. The amphoterin/RAGE-mediated effect on process extension would thus be transient, as expected during cell motility, and would be coupled to proteolytic activation enhancing penetration of a process in tissue.

The ability of malignant cells to infiltrate structures that are adjacent to or distant from the primary tumor site is an important determinant of the poor prognosis in tumors. The infiltrative behavior requires migration and invasion, which in addition to locomotion involves proteolytic activities to degrade the extracellular matrix. Amphoterin regulates cell migration, as shown herein, and enhances proteolytic activities (Parkkinen et al., 1991). Therefore, the regulation of cell migration by amphoterin should be of interest in invasive behaviour of tumor cells. Further, amphoterin may regulate developmental phenomena, like the invasive phenotype of migrating growth cones during the development of neuronal connections.

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